REMARKS

Claims 54-63 and 65-103 are pending in the application. All claims were rejected in the Final Office Action of April 3, 2008.

I. Interview Summary

Applicants respectfully thank Examiners Hissong and Landsman for the courtesy of a telephone interview with the undersigned and Dr. Ann-Louise Kerner on September 9, 2008. The Examiners indicated that arguments as presented herein should be helpful in overcoming the outstanding written description and enablement rejections.

II. Specification

The Office Action states that the specification is objected to because the Examiner's copy of page 27 was not reproduced legibly in certain sections. Applicants submit herewith a duplicate copy of page 27 of the application as filed, thus addressing this objection. Applicants note that this copy of page 27 was obtained from the copy of PCT application PCT/EP00/00598 that was submitted with the present application as part of the US national stage filing, as reflected on private PAIR.

III. Claim Rejection Under 35 U.S.C. § 112, 1st Paragraph – Enablement

Claims 54-63 and 65-103 were rejected under § 112, first paragraph, as lacking enablement, because the specification allegedly does not provide sufficient support for selection of surfactants and lipids suitable for use in the claimed methods. In particular, the Final Office Action expressed concern that, although the specification describes phosphatidylcholines as useful in the claimed penetrants, Applicants distinguished a certain didecanoyl-L-alpha-phosphatidylcholine absorption enhancer disclosed in the previously-cited Drejer reference ("Drejer phosphatidylcholine") as unlikely to function as claimed. Applicants respectfully traverse this rejection.

As described in more detail in Applicants' Reply to the Office Action of October 10, 2007, the specification and claims provide ample information on how to prepare suitable penetrant compositions with lipids and surfactants as claimed. Briefly, independent claims 54 and 100 explicitly recite the functional properties of a suitable penetrant, and this functional claim language ensures that the claims do not read on inoperative embodiments. In any case, even a claim encompassing some inoperative embodiments is not invalid for lack of enablement

if one skilled in the art could determine the operative embodiments without undue experimentation. *See* MPEP 2164.08(b), citing *Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 1569, 1577 (Fed. Cir. 1984). Here, the specification (pages 26-28) lists various lipids and surfactants suitable for use in the claimed penetrants, and also provides guidance as to the selection of suitable materials. The specification describes and exemplifies the preparation of penetrant compositions, thus allowing one skilled in the art to prepare compositions as claimed (and identify unsuitable compositions) without undue experimentation. *See, e.g.*, page 40, second and third paragraphs, and subsequent Examples; page 16, first paragraph; and page 13, fourth and fifth paragraphs (citing additional references).

The Declaration of Dr. Gregor Cevc submitted on June 28, 2007 shows how one skilled in the art could readily evaluate the operative embodiments according to the functional characteristics identified in the claims and specification. *See, e.g.*, Declaration, ¶¶ 10-15. Dr. Cevc's Declaration explains the important role of the shape and form of the claimed penetrant composition for achieving an effective transnasal administration as claimed. *See, e.g.*, Declaration, §14. In particular, penetrants as claimed result in "ultradeformable lipid vesicles" (Declaration, §9) comprising a fluid droplet with a coating of at least two substances that differ by at least a factor of 10 in solubility, the two substances forming aggregates with specified diameter limitations, the more soluble substance solubilizing the droplet, and/or the coated droplet having a particularly described elastic deformation energy. Independent claims 54 and 100 further recite that "the **less soluble substance** is a lipid and the **more soluble substance** is a surfactant or more soluble form of the lipid." All of these features collectively achieve the disclosed efficient transfer of the composition across a transnasal barrier. Declaration, §10.

As discussed with the Examiners, Dr. Cevc's Declaration explains that "the 'Drejer phosphatidylcholine' cannot form large bilayer vesicle aggregates, as is requested in the Application, but rather self-assembles into small aggregates in micellar form, **owing to is *[sic]* too high water solubility**." Declaration, §11 (emphasis added). As shown by the attached Exhibit A, one having ordinary skill in the art would have been aware that the "Drejer phosphatidylcholine" has higher water solubility due to its shorter (C_{10}) acyl chain length. Exhibit A, Vinggaard et al., *Biochem. J.* **319**:861-864 (1996) (page 864, second column, stating that "C_{10}-PC [didecanoyl phosphatidylcholine] is less hydrophobic than the longer-chain PCs"). Thus, one skilled in the art would realize that the **more soluble** "Drejer phosphatidylcholine"

would not be suitable for arriving at a penetrant composition having the characteristics as claimed.

Furthermore, the known **high water solubility** of the "Drejer phosphatidylcholine" would also be considered by one skilled in the art to be undesirable for the claimed penetrant in view of explicit description provided in the specification as filed:

> In an additional preferred embodiment of the use or of the pharmaceutical composition of the present invention the **less soluble self-aggregating molecule is a lipid**, preferably a polar lipid, and the more soluble component is a surfactant or some more soluble form of the polar/basic lipid. The former ingredient [*i.e.*, the less soluble self-aggregating molecule], typically, stems from a biological source or is a corresponding synthetic lipid or any of its modifications. ... or any other bilayer forming lipids, and preferably is selected from the group of phosphatidylcholines ... Specification, pages 26-27 (emphasis added).

One skilled in the art consulting the above text in the specification, in view of common general knowledge (*e.g.*, Exhibit A), would not reasonably consider using the more hydrophilic (thus *not* "less soluble") "Drejer phosphatidylcholine" to prepare a penetrant as claimed. Instead, he would immediately realize that that this phospholipid species has solubility characteristics that are not useful for achieving the large bilayer vesicle aggregates / ultradeformable lipid vesicle form that provides efficient transnasal delivery across the nasal mucosa membranes.

In sum, the extensive guidance provided in the specification and claims, informed by the knowledge in the field, ensures that a person of ordinary skill in the art would readily be able to practice the claimed invention without undue experimentation. Thus, Applicants respectfully submit that the present enablement rejection should be reconsidered and withdrawn.

IV. Claim Rejection Under 35 U.S.C. § 112, 1st Paragraph – Written Description

Claims 54-63 and 65-103 were rejected under § 112, first paragraph, for allegedly lacking sufficient written description. The Final Office Action again expressed concern regarding the sufficiency of disclosure of functional lipids to form the claimed penetrants, in view of the inoperative "Drejer phosphatidylcholine." Applicants respectfully traverse this rejection.

Written description for a claimed genus is satisfied by providing sufficient description of a representative number of species within the genus, and/or identifying characteristics such as structure, physical properties, chemical properties, and/or functional characteristics. *See* MPEP 2163, citing *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 1568 (Fed. Cir.

1997), *cert. denied*, 523 U.S. 1089 (1998). Applicants respectfully submit that the present claims and specification satisfy this standard. As described in Applicants' Reply to the Office Action of October 10, 2007, the claims' recitation of "lipids" and "surfactants" itself conveys a discrete group of compounds having common structural elements, physical and chemical properties that are well-understood by those skilled in the art. Moreover, independent claims 54 and 100 additionally set forth a detailed group of functional characteristics that clearly describe multiple features of the lipid and surfactant substances that form a suitable penetrant. These features are also identified in the body of the specification, for example, in the paragraph bridging pages 12-13.

In addition to the description in the claims themselves, the specification provides ample support for the claimed penetrant including a lipid and a surfactant or more soluble form of the lipid. The specification at pages 26-28 lists various lipids and surfactants suitable for use in the claimed penetrants, identifying these materials by chemical names and/or structures. The specification also discusses and exemplifies the preparation of penetrant compositions. *See, e.g.*, page 40, second and third paragraphs, and subsequent Examples; page 16, first paragraph; page 13, fourth and fifth paragraphs (citing additional references).

The Final Office Action expressed concern that although the specification describes phosphatidylcholines as useful in the claimed penetrants, Applicants distinguished the "Drejer phosphatidylcholine" as unlikely to function as claimed. However, the fact that a category of lipids generally described as useful includes an inoperative material does not render Applicants' written description insufficient. To the contrary, one skilled in the art would understand that in addition to listing categories of potentially useful materials, Applicants further defined the genus of useful lipids and surfactants based on functional characteristics. These characteristics are set forth in the specification and claims, and are demonstrated in the working Examples (which also exemplify particular combinations of materials that form conventional liposomes or micelles instead of penetrants as claimed). As discussed with the Examiners and explained above, the discussion of materials qualifying for the claimed **"more soluble substance"** and **"less soluble substance"** clearly provides the skilled person with sufficient description to both (a) produce the functional characteristics of the composition recited in claims 54 and 100, and (b) differentiate functional lipids from those that would not be functional in the claimed penetrant composition.

As discussed in more detail above, the Declaration of Dr. Gregor Cevc submitted on June 28, 2007 describes the types of observations that one skilled in the art would readily make to appreciate which materials do or do not exhibit the claimed functional characteristics. *See, e.g.*, Declaration, ¶¶ 10- 15. For example, Dr. Cevc's Declaration explains that "the 'Drejer phosphatidylcholine' cannot form large bilayer vesicle aggregates, as is requested in the Application, but rather self-assembles into small aggregates in micellar form, **owing to is *[sic]* too high water solubility**." Declaration, §11 (emphasis added). As noted above, the high water solubility of the "Drejer phosphatidylcholine" would be understood by one skilled in the art, for example, as shown by the attached Exhibit A.

In sum, the specification and claims, in view of the knowledge of one having ordinary skill in the art, clearly provide sufficient written description to support the claimed invention. Accordingly, Applicants respectfully submit that the present written description rejection should be reconsidered and withdrawn.

V. Conclusion

In view of the arguments set forth above, Applicants respectfully submit that the objections and rejections contained in the Final Office Action mailed on April 3, 2008 have been overcome, and that the pending claims are in condition for allowance.

Applicants request a three-month extension of time for responding to the Final Office Action of April 3, 2008. Please charge the $1,110.00 extension fee, along with the $810.00 fee for the present Request for Continued Examination to our Deposit Account No. 08-0219. No other fees are believed to be due in connection with this correspondence. However, please charge any payments due or credit any overpayments to our Deposit Account No. 08-0219.

The Examiner is encouraged to telephone the undersigned at the number listed below in order to expedite the prosecution of this application.

Respectfully submitted,

Dated: 10/2/08

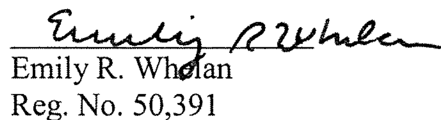

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EXHIBIT A

Biochem. J. (1996) 319, 861–864 (Printed in Great Britain)

Didecanoyl phosphatidylcholine is a superior substrate for assaying mammalian phospholipase D

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Phospholipase D (PLD) activity in crude or solubilized membranes from mammalian tissues is difficult to detect with the current assay techniques, unless a high radioactive concentration of substrate and/or long incubation times are employed. Generally, the enzyme has to be extracted and partially purified on one column before easy detection of activity. Furthermore, PLD activity in cultured cells can only be detected by the available assay techniques in the presence of guanosine 5′-[γ-thio]-triphosphate (GTP[S]) and a cytosolic factor [usually ADP-ribosylation factor (Arf)]. In this paper we report that the use of didecanoyl phosphatidylcholine (C_{10}-PC) in mammalian PLD assays considerably increases the detection limit. C_{10}-PC was compared with the commonly used dipalmitoyl phosphatidylcholine (C_{16}-PC) as a substrate for PLD activity from membranes of human neutrophils, human placenta and pig brain, and from placental cytosol. C_{10}-PC was superior to C_{16}-PC by a factor of 2–28 depending on assay conditions and tissue, and it allowed the detection of GTP[S]-and Arf-stimulated PLD activity without addition of phosphatidylinositol 4,5-bisphosphate.

INTRODUCTION

Activation of phospholipase D (PLD) is involved in the signal transduction of both seven-membrane-spanning receptors activated by hormones and tyrosine kinase receptors activated by growth factors [1]. Generally, measurement of PLD activity has been done either by using cells endogenously radiolabelled in their phospholipids or by using exogenous radiolabelled phospholipid substrate. However, PLD in crude or solubilized membranes from mammalian tissues is difficult to detect with the current assay techniques, unless high amounts of radioactive substrate and long incubation times are employed [2] and/or ammonium sulphate is added [3]. Thus membranes from mammalian brain and several other tissues (e.g. rat lung and bovine kidney) showed very low PLD activities in both the presence and the absence of Arf (ADP-ribosylation factor) and guanosine 5′-[γ-thio]triphosphate (GTP[S]) [4]. In general, the PLD activity of a number of tissues is not detectable until membranes are extracted with detergent and subjected to an initial chromatography step [5]. In addition, the PLD activity of membranes from cell lines (including HL-60 cells) is not readily observed (even in the presence of GTP[S]) unless a cytosolic factor such as Arf is added [4,6,7]. The low activities observed in tissues and some cell lines have been suggested to be due to factors that interfere with the assay of PLD [4]. Furthermore, it was found that the inclusion of phosphatidylinositol 4,5-bisphosphate (PIP_2) was an absolute requirement for effective measurement of Arf-stimulated PLD activity [5].

In this paper we report on the benefits of a new substrate for PLD, i.e. phosphatidylcholine (PC) containing two C_{10} fatty acids. Didecanoyl PC (C_{10}-PC) is superior to long-chain PCs by a factor of 2–28 depending on assay conditions and tissue. The use of this substrate allows for easy screening of tissues and cell lines for PLD activity without the need to add GTP[S], cytosolic factors such as Arf or Rho, or high salt concentrations. Even addition of PIP_2 may be omitted when measuring Arf-stimulated PLD. The use of C_{10}-PC as an exogenous substrate in PLD assays may greatly facilitate the characterization and purification of this enzymic activity.

MATERIALS AND METHODS

Materials

1,2-[^{14}C]Didecanoyl-*sn*-glycero-3-phosphocholine (67 mCi/mmol) and 1,2-didecanoyl-*sn*-glycero-3-phospho[^{3}H]choline (85 Ci/mmol) (prepared by Amersham) were gifts from Novo Nordisk. 1-Stearoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (54 mCi/mmol), 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine (57 mCi/mmol) and 1-palmitoyl-2-[^{14}C]-palmitoyl-*sn*-glycero-3-phosphocholine were from Amersham, and 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (57 mCi/mmol) was from Dupont–NEN. 1,2-Dipalmitoyl-*sn*-glycero-3-phospho[^{3}H]choline was obtained from NEN (Figure 1) and Amersham (Figure 3). Glass-backed silica gel 60 TLC plates were from Merck (Darmstadt, Germany). Ecoscient scintillation fluid was from National Diagnostics. Dithiothreitol, Triton X-100 and octyl glucoside were from Boehringer (Mannheim, Germany). Phosphatidylethanolamine (PE; from egg yolk) and all other chemicals were from Sigma (St. Louis, MO, U.S.A.). Recombinant myristoylated Arf (mArf1) was kindly provided by Dr. S. Paris (CNRS, Valbonne, France), and recombinant non-myristoylated Arf (rArf1) was prepared as described previously [8].

Preparation of placental membranes, placental cytosol and pig brain membranes, and fractionation of placental proteins

Human placentae were homogenized as described previously [9]. Briefly, the tissue was homogenized with an UltraTurrax® for

Abbreviations used: Arf, ADP-ribosylation factor; mArf1, recombinant myristoylated Arf; rArf1, recombinant non-myristoylated Arf; GTP[S], guanosine 5′-[γ-thio]triphosphate; PC, phosphatidylcholine; C_{10}-PC, didecanoyl PC; C_{16}-PC, dipalmitoyl PC; PE, phosphatidylethanolamine; PEt, phosphatidylethanol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D.

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3 min in 2 vol. of buffer A (50 mM Hepes, pH 7.0, 1 mM EDTA, 1 mM PMSF and 1 mM dithiothreitol). After centrifugation at 500 *g* for 10 min at 4 °C, the supernatant was centrifuged at 109000 *g* for 60 min at 4 °C. The supernatant and pellet were designated 'cytosol' and 'membranes' respectively. The membrane fraction was suspended in buffer A plus 0.15 M NaCl and 1 % Triton X-100 (buffer B). The suspension was agitated for 60 min at 4 °C and then centrifuged at 109000 *g* for 60 min at 4 °C. The supernatant was diluted 10 times with buffer B with no Triton X-100 for column chromatography (giving a final concentration of 0.1 % Triton X-100) or for measurement of PLD activity. Solubilized proteins and cytosol were purified on DEAE-Sepharose columns as described ([9,9a]. Fractionation of the cytosolic enzyme employed the same procedure, except that no Triton X-100 was added to the buffers. Solubilized proteins from pig brain membranes were prepared according to the above procedure. Protein was determined by the Bradford procedure, using IgG as a standard.

Preparation and fractionation of PLD activity from human neutrophil membranes

Human neutrophils were prepared from human blood. They were then allowed to swell in hypotonic buffer and cell-cracked. A post-nuclear supernatant was prepared by low-speed centrifugation and applied to a continuous 10.4–40 % sucrose gradient with a 65 % cushion. Fractions were assayed for PLD and marker enzymes. The peak activity associated with plasma membrane markers was used for testing of the substrate.

Determination of PLD activity in placental and brain tissues

PLD activity was measured by monitoring the release of [^{3}H]choline according to [10] with minor modifications. Incubations in a total volume of 100 μl were carried out for the indicated time periods at 37 °C. [^{3}H]C_{10}-PC or [^{3}H]dipalmitoyl phosphatidylcholine ([^{3}H]C_{16}-PC) and C_{10}-PC or C_{16}-PC were added, and the solvents were evaporated under a stream of N_2. Vesicle formation was carried out by sonication for 10 min with 50 mM Hepes and 0.07 % octyl glucoside. The exact assay conditions, including substrate concentrations, added buffers and salts, are specified in the legends to the Figures. Incubations were stopped by addition of 50 μl of BSA (20 mg/ml) and 100 μl of trichloroacetic acid (20 %) followed by centrifugation at 10000 *g* for 10 min at 4 °C. An aliquot (200 μl) of the supernatant was counted for radioactivity by liquid scintillation counting.

Measurement of phosphatidylethanol (PEt) formation using ^{14}C-labelled PCs was done essentially as described previously [9]. The exact assay conditions, including substrate composition, added buffers and salts, are specified in the text and legend to the Figures. NaF was included as an inhibitor of phosphatidic acid phosphatase. The reactions were stopped by addition of 2 ml of chloroform/methanol/conc. HCl (200:100:0.5, by vol.). The lipids were extracted and separated on TLC plates as described [9]. The activity values in the Figures were obtained by subtracting the control value.

Determination of PLD activity in human neutrophil membranes

Substrate was prepared from PE, PIP_2 and PC (C_{10} or C_{16}) in the molar ratio 10:0.3:1. For vesicles not containing PIP_2, the vesicle composition was PE/PC (10:1, mol/mol). The final PC concentration was 8.6 μM. [^{3}H]Choline-labelled PC was included to give approx. 50000 d.p.m. per 10 μl of substrate. Assays were performed at 37 °C with 1.5–2.0 μg of protein for 60–90 min. A 10 μl aliquot of substrate was added in a final volume of 60 μl. mArf1 was used at 40 μM, and GTP[S] at 30 μM. Substrate and assay buffer was 50 mM Na/Hepes (pH 7.5), 3 mM EGTA, 80 mM KCl and 1 mM dithiothreitol, with the inclusion of $MgCl_2$ (0.5 mM) and $CaCl_2$ (2 mM) in the assay buffer. [^{3}H]Choline was extracted and measured as described previously [11].

RESULTS

Experiments were performed in order to compare [^{14}C]C_{10}-PC with three other molecular species of [^{14}C]PC containing the fatty acids $C_{16:0}/C_{18:1}$, $C_{16:0}/C_{20:4}$ and $C_{18:0}/C_{20:4}$. Incubations were run for 10 min with 1 mM phospholipid (0.05 μCi/assay) and 40 μM PIP_2. To our surprise, no PLD activity (measured by PEt formation) was detected using PCs containing relatively long-chain fatty acids, whereas the relatively short-chained C_{10}-PC allowed easy detection of extracted/purified PLD from human placenta. Thus the PLD activities (PEt formation) of solubilized membranes (117 μg of protein) and of DEAE-Sepharose peak fractions of membranes (110 μg) and cytosol (168 μg) were 3731 ± 481 (mean ± range, $n = 2$), 7754 ± 613 and 192 ± 25 pmol/min per mg of protein respectively.

Next, C_{10}-PC was compared with C_{16}-PC, the most commonly used exogenous substrate in PLD assays. Increasing concentrations of C_{10}-PC or C_{16}-PC (0.1–4 mM) were added to cytosolic PLD from human placenta that had been partially purified on a DEAE-Sepharose column (Figure 1). Cytosolic PLD was chosen as the PLD source in these experiments. This PLD offers an advantage over membrane PLD as no detergents have to be added, and thus the mol % of substrate in the vesicles can be kept constant, which means that considerations of surface dilution can be ignored. The PIP_2 level was kept constant at 3.8 mol %.

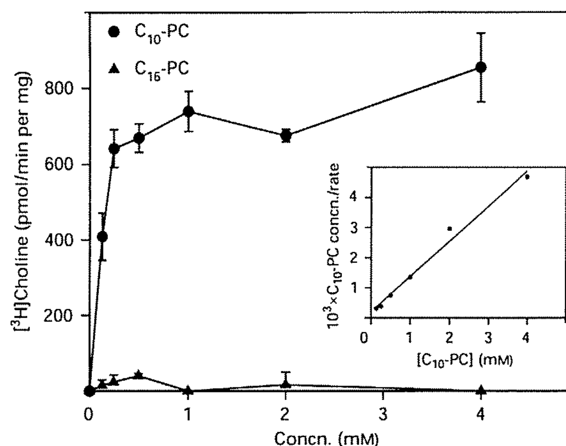

Figure 1 Effect of increasing concentrations of C_{10}-PC or C_{16}-PC on the activity of partially purified cytosolic PLD from human placenta

Cytosol from human placenta was partially purified on a DEAE-Sepharose column. A sample of the PLD peak fraction (216 μg of protein) was incubated with 0.13–4 mM [^{3}H]C_{10}-PC (2000 d.p.m./nmol) or [^{3}H]C_{16}-PC (3600 d.p.m./nmol), 3.8 mol% PIP_2, 0.01% octyl glucoside, 50 mM Hepes, pH 7.5, 2 mM $CaCl_2$, 3 mM $MgCl_2$, 3 mM EGTA, 80 mM KCl and 1 mM dithiothreitol for 15 min at 37 °C. Release of [^{3}H]choline was measured as described in the Materials and methods section. The insert shows a Hanes–Woolf transformation of the data obtained with C_{10}-PC. Data represent the means ± ranges of the results of one experiment in duplicate performed twice.

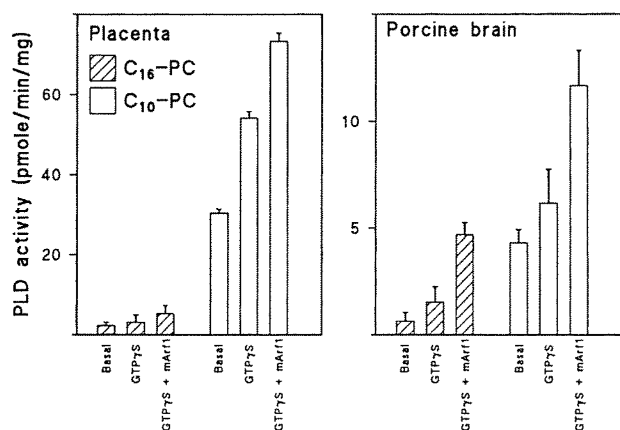

Figure 2 Comparison of C_{10}-PC and C_{16}-PC as substrates for PLD from membranes of human placenta and pig brain

Left panel: substrate vesicles of PE/PIP_2/PC (600:30:60 μM) containing [^{14}C]C_{10}-PC (18000 d.p.m./nmol) or [^{14}C]C_{16}-PC (21000 d.p.m./nmol) were incubated with Triton X-100-solubilized membrane proteins from human placenta (64 μg of protein) for 30 min. The vials contained 50 mM Hepes, pH 7.5, 3 mM EGTA, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM dithiothreitol, 80 mM KCl and 15 mM NaF in a final volume of 100 μl. The membrane proteins were incubated alone, with GTP[S] (GTPγS; 20 μM) or with GTP[S] plus mArf1 (2.5 μM). Lipids were extracted and assayed for [^{14}C]PEt formation by TLC. Data represent the means ± ranges of one experiment in duplicate performed twice. Right panel: solubilized membrane proteins from pig brain (85 μg of protein) were incubated and product formation measured as described for placental proteins.

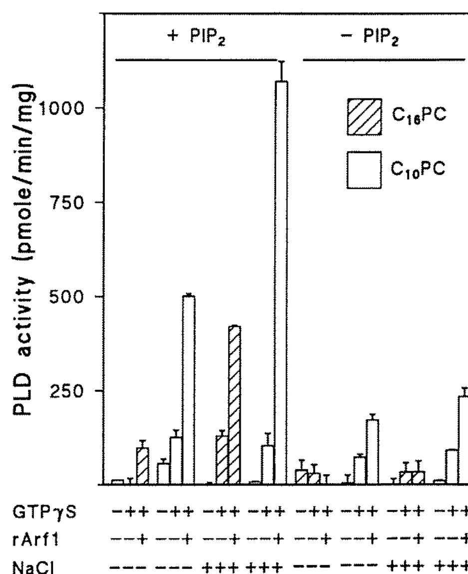

Figure 3 Comparison of C_{10}-PC and C_{16}-PC as exogenous substrates for a partially purified PLD fraction from human neutrophil membranes

Neutrophil plasma membranes were incubated with substrate vesicles composed of PE/PIP_2/PC (10:0.3:1 μM) or PE/PC (10:1 μM) containing [^{3}H]choline-labelled C_{10}-PC or C_{16}-PC. The final PC concentration was 8.6 μM. The vials were incubated with 1.5–2.0 μg of membrane protein for 90 min. The PLD fractions were incubated alone, with 30 μM GTP[S] (GTPγS) and/or with 40 μM rArf1, in the presence or absence of 0.5 M NaCl. Data represent the means ± ranges of one experiment in duplicate performed twice. A third experiment with PE/PIP_2/PC (86:2.6:8.6 μM) was performed for t = 60 min with similar results.

Using C_{10}-PC the enzyme followed Michaelis–Menten kinetics, with a K_m of approx. 0.1 mM. Transforming the data to a Hanes–Woolf plot [12], which ensures a more uniform distribution of the data points, resulted in a straight line. However, with C_{16}-PC, very low, if any, PLD activity was observed, and it was not possible under these assay conditions (see the legend to Figure 1) to obtain a Michaelis–Menten curve. In this experiment C_{10}-PC was superior to C_{16}-PC in the concentration range 0.13–0.5 mM by a factor of 17–28.

In order to optimize the assay employing C_{16}-PC, the assay conditions were changed to those described in the legend to Figure 2. Comparison of C_{10}-PC and C_{16}-PC under these assay conditions showed that PLD activities in membranes from placenta and brain were measurable with both substrates. However, pronounced differences in PLD activities obtained using C_{10}-PC compared with C_{16}-PC as substrate were observed with membranes both from human placenta (by a factor of 15) (Figure 2, left panel) and from pig brain (by a factor of 4) (Figure 2, right panel). The addition of GTP[S] (20 μM) or mArf1 (2.5 μM) did not change this pattern. Generally, the specific activity of PLD was higher in membranes from human placenta than in those from pig brain. However, this difference was more pronounced using C_{10}-PC compared with C_{16}-PC.

In order to extend the study, PLD activity in human neutrophils was measured. The PLD activity was from a highly enriched fraction of plasma membranes, purified on a continuous sucrose gradient. Results showed that, for this PLD also, C_{10}-PC was excellent as an exogenous substrate compared with C_{16}-PC, irrespective of whether GTP[S], rArf1 or 0.5 M NaCl was added (Figure 3). NaCl (0.5 M) was found to activate neutrophil PLD activity. Omitting PIP_2 from the incubations resulted in lower, but still measurable, activity with C_{10}-PC, whereas C_{16}-PC was not a very useful substrate under these assay conditions (Figure 3).

Current PLD assay techniques involve the use of C_{16}-PC together with PIP_2 and PE as substrate [2,5]. While the reason for adding PIP_2 is obvious, the reason for adding PE has not been clarified. Our experiments showed that the addition of PE (egg yolk), giving a phospholipid composition of PE/PIP_2/PC of 600:15:30 μM, resulted in a decrease in the basal placental membrane PLD activity purified on a DEAE-Sepharose column, measured in the absence of Arf and GTP[S] (results not shown). This was the case with either C_{16}-PC or C_{10}-PC as substrate (2-fold decrease in both cases; means for n = 4). However, using substrate compositions of 86:2.6:8.6 μM or 100:15:10 μM, the addition of PE with C_{16}-PC resulted in an almost unchanged response. Addition of phosphatidylserine, which is thought to have no effect on PLD activity, gave rise to a decreased PLD response in all cases.

DISCUSSION

The ability to obtain a pure mammalian PLD by protein purification or molecular biological techniques is of major importance to the area of agonist-stimulated PC hydrolysis. Until now, only one report of the purification to homogeneity of a mammalian PLD has appeared, namely an oleate-activated form of PLD that was purified from pig lung [13]. Recently, an Arf-stimulated PLD was highly purified from pig brain to a specific activity of 28 nmol/min per mg of protein, as determined with C_{16}-PC in the presence of Arf [4]. There has also been a recent report on the cloning of Arf-stimulated PLD from HeLa cells [14]. However, the purification of Arf-stimulated PLD to homogeneity still remains to be done. The major obstacle to this

project is that PLD is labile during purification, and that cytosolic factors have to be added for detection of PLD activity [4].

Here we describe PLD activity towards a short-chained PC containing two C_{10} fatty acids, the use of which results in a pronounced increase in sensitivity of the assay. Using optimum assay conditions (1 mM C_{10}-PC), specific PLD activities in human placental membranes can be measured in the range of nmol/min per mg. For comparison, the reported specific activities of both cytosolic and membrane-associated enzymes from brain determined in the presence of C_{16}-PC are approx. 16 pmol/min per mg of protein [6]. In contrast, the PLD specific activity in agonist-stimulated cells such as neutrophils is around 4.5 nmol/min per mg [6]. Furthermore, we have shown that PLD activities in human neutrophil membranes can be measured with C_{10}-PC in the absence of PIP_2. Previously we have found that membrane PLD activities in human placenta are also easily measured using our substrate without adding PIP_2 [9].

The assay conditions in the present paper were varied between the different experiments. As the time curve for cytosolic placental PLD measured with C_{10}-PC is only linear for 15–20 min (A. M. Vinggaard, T. Jensen and H. S. Hansen, unpublished work), this incubation period was chosen for the Michaelis–Menten curve. In our hands, virtually no PLD activity towards C_{16}-PC can be measured under these conditions. Thus the optimum assay conditions for measuring PLD activity towards C_{10}-PC do not allow measurement of any activity with C_{16}-PC. We think that it is a strength of our results that, irrespective of which assay conditions are used, C_{10}-PC is under all circumstances the best substrate, even if the optimum conditions for measuring activity with this substrate have not been met. Thus the purpose of the application of different assay conditions is to show that C_{10}-PC will be superior under all of them. Furthermore, we have used C_{10}-PC in the standard assays of PLD from two independent laboratories (i.e. in Copenhagen and London).

The composition of the lipid vesicles has been reported to be crucial for observing guanine nucleotide-sensitive hydrolysis of PC by HL-60 membranes and partially resolved activities derived from these membranes [10]. Thus a substrate composed of PE/PIP_2/PC at 100:15:10 μM was recommended [5]. Our experiments with placental membrane PLD show that the addition of PE to the substrate vesicles in the absence of Arf and GTP[S] resulted in a lower or unchanged PLD activity, irrespective of using C_{10}-PC or C_{16}-PC as substrate; however, C_{10}-PC was still superior to C_{16}-PC. Recently it was reported that PE containing at least one unsaturated fatty acid was able to dramatically enhance the activity of partially purified PLD from bovine kidney membranes towards C_{16}-PC [15]. Activity was measured in the presence of small G-proteins, GTP[S] and ammonium sulphate. Thus it may be that PE is important for G-proteins to stimulate PLD, whereas it may not affect basal PLD activity.

The kinetics of phospholipase action against aggregated substrates can be divided into two parts: (1) binding of the enzyme to the aggregated substrate (interfacial phenomena), and (2) kinetic processing of the substrate [16]. Determining whether an altered activity is the result of changed interfacial binding or kinetic processing can be extremely difficult [16]. The reason for the excellent substrate properties of C_{10}-PC is not known at present. C_{10}-PC is less hydrophobic than the longer-chain PCs, having a critical micellar concentration of 5.0 μM [17]. As the PLD activity increases with increasing substrate concentrations above the critical micellar concentration, where the monomeric concentration is constant, the free monomer C_{10}-PC does not seem to be the substrate for PLD. Studies of phospholipid hydrolysis by phospholipase A_2 (*Naja naja naja*) and phospholipase C (*Bacillus cereus*) have shown that short-chain PCs in a micellar matrix are excellent substrates for both of these enzymes [16]. This has been suggested to be due to relatively weak intermolecular interactions in the micelle. The greater mobility and lack of interlipid interactions may make the short-chain PCs more 'accessible' to phospholipases. Since less energy is required to disrupt phospholipid/phospholipid packing interactions, either easier extraction/removal of the PC from the vesicle or better binding of PC to the enzyme may be involved in the mechanism of action [18]. These mechanisms may also apply to the hydrolysis of short-chain PCs by PLD.

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where R_1 and R_2 is an aliphatic chain, typically a C_{10-20}-acyl, or -alkyl or a partly unsaturated fatty acid residue, in particular, an oleoyl-, palmitoeloyl-, elaidoyl-, linoleyl-, linolenyl-, linolenoyl-, arachidoyl-, vaccinyl-, lauroyl-, myristoyl-, palmitoyl-, or stearoyl chain, and where R_3 is hydrogen, 2-trimethylamino-1-ethyl, 2-amino-1-ethyl, C_{1-4}-alkyl, C_{1-5}-alkyl substituted with carboxy, C_{2-5}-alkyl substituted with hydroxy, C_{2-5}-alkyl substituted with carboxy and hydroxy, or C_{2-5}-alkyl substituted with carboxy and amino, inositol, sphingosine, or salts of said substances, said lipid comprising also glycerides, isoprenoid lipids, steroids, sterines or sterols, of sulphur- or carbohydrate-containing lipids, or any other bilayer forming lipids, and preferably is selected from the group of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids, phosphatidylserines, sphingomyelins or other sphingophospholipids, glycosphingolipids (including cerebrosides, ceramidepolyhexosides, sulphatides, sphingoplasmalogens), gangliosides or other glycolipids or synthetic lipids, in particular with corresponding sphingosine derivatives, or any other glycolipids, whereby two similar or different chains can be esterified to the backbone (as in diacyl and dialkenoyl compound) or be attached to the backbone with ether bonds, as in dialkyl-lipids, or belong to the backbone as in sphingolipids.

The surfactant used, normally, is nonionic, zwitterionic, anionic or cationic, especially a fatty-acid or -alcohol, an alkyl-tri/di/methyl-ammonium salt, an alkylsulphate salt, a monovalent salt of cholate, deoxycholate, glycocholate, glycodeoxycholate, taurodeoxycholate, taurocholate, etc., an acyl- or alkanoyl-dimethyl-aminoxide, esp. a dodecyl- dimethyl-aminoxide, an alkyl- or alkanoyl-N-methylglucamide, N-alkyl-N,N-dimethylglycine, 3-(acyldimethylammonio)-alkanesulphonate, N-acyl-sulphobetaine, a polyethylene-glycol-octylphenyl ether, esp. a nonaethylene-glycol-octylphenyl ether, a polyethylene-acyl ether, esp. a nonaethylen-dodecyl ether, a polyethylene-glycol-isoacyl ether, esp. a octaethylene-glycol-isotridecyl ether, polyethylene-acyl ether, esp. octaethylenedodecyl ether, polyethylene-glycol-sorbitane-acyl ester, such as polyethylenglykol-20-monolaurate (Tween 20) or polyethylenglykol-20-sorbitan-monooleate (Tween 80), a polyhydroxyethylene-acyl ether, esp. polyhydroxyethylene-lauryl, -myristoyl, -cetylstearyl, or -oleoyl ether, as in polyhydroxyethylene-4 or 6 or 8 or 10 or 12, etc., -lauryl ether (as in Brij series), or in the corresponding ester, e.g. of polyhydroxyethylen-8-stearate (Myrj 45), myristate-, -laurate, linoleate-, linolenate-, palmitoleate- or -oleate type, or in polyethoxylated castor oil 40, a sorbitane-monoalkylate (e.g. in Arlacel or Span), esp. sorbitane-monolaurate, -myristate,